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Abstract

The development of any organism is directed by sequential gene expression that provides a basis for differentiation and specialization of cells. Our lab is interested in the genes that control organogenesis, specifically, the development of the *C. elegans* pharynx. The microscopic nematode *Caenorhabditis elegans* is a model organism that provides many advantages for this study, including a completely sequenced genome, a known cell lineage and a transparency that makes observation very simple. We selected two strains from a previous mutagenesis screen to investigate, M136 and M138, which are characterized by extreme deformation of the pharynx resulting from possible failure of pharyngeal muscle cells to undergo normal morphogenesis. We hypothesized that the phenotype may be a result of abnormal adhesion of cells and resulting in L1 larval lethality. We addressed the basis of this lethality using multiple methods, immunocytochemistry to reveal the structure of the abnormal pharynx, fluorescent bead feeding assays to determine if the pharynx has function, genetic mapping to reveal the identity of the gene involved, and a genetic cross to determine the presence of the correct number of cells. Single nucleotide polymorphism mapping revealed the mutation to be located on chromosome I between map units one and eight. A complementation analysis showed M136 and M138 are caused by a mutation to the same gene. Immunocytochemistry demonstrated the mutation caused defects to multiple cell types and but the genetic cross indicated all pharynx muscle cells are present in mutant worms. Future research will entail complementation with deletion strains to determine the exact location of the mutation, followed by the use of transgenic rescue microinjections to confirm the identity of the gene.

Introduction

The field of developmental biology seeks to explain the fascinating sequence of events that allows a single cell to develop into a complex, functional organism. Nearly every eukaryote organism begins with the fusion of a sperm and an egg to create a diploid cell, called a zygote. The subsequent division, differentiation, specialization and growth of this cell over hours, days or months will ultimately result in an organism such as ourselves. Developmental biology is a field of far-reaching implications and research within it has led to the understanding of numerous birth defects, and to the development of the innovative techniques involved in procedures such as *in vitro* fertilization (Slack, 2005). Furthermore, it has become clear that the period of organogenesis is particularly critical during embryogenesis, a time in which any alteration of normal development can result in significant birth defects.

Our lab focuses on the process of organogenesis, in particular, the genetic regulation that orchestrates the development of an organ from a single cell. We use an organ known as the pharynx of the microscopic worm *Caenorhabditis elegans* as our model in this study. Using a forward genetics approach, we are looking to identify the genes causing mutant phenotypes in the *C. elegans* pharynx to assemble a pathway for normal pharynx development.

Differentiation and Cell Fate

How does a single egg, upon fusion with sperm, generate countless differentiated cells that perform diverse functions? At some point, each cell in the embryo adopts a particular fate, meaning it can only specialize into one type of cell. There are several factors that influence cell fate commitment, such as the contribution of maternal RNA, zygotic gene regulation and intercellular signaling (Slack, 2006). Maternal RNA is RNA passed to the embryo by their mother during oogenesis. Both vertebrate and invertebrate zygotes use maternal RNA to provide the first instructions for their development. For example, in Drosophila melangaster, commonly known as the fruit fly, the maternal RNA nanos is localized to the posterior pole of the oocyte. The resulting gradient of translated Nanos protein contributes to the development of the anterior-posterior axis, and stimulates expression of later zygotic genes (Curtis et al., 1995)

The activation and repression of different sets of zygotic genes, also known as differential gene expression, plays a huge role in the differentiation and specialization of cells during embryogenesis. Differential gene expression is responsible for increasingly restricting cell fate as development progresses. The founder cell in a zygote is totipotent; it can differentiate into any different cell type, including extraembryonic tissue (Slack, 2005). Early fate restrictions produce pluripotent cells that have the ability to commit to all three germ layers in an organism, but not the extraembryonic tissue. They divide into multipotent cells, which are committed to a subset of specialized cell fate (Slack, 2005).

In *C. elegans*, the gene pha-4 is expressed in all pharynx cells and is considered to be an organ identity gene. Without pha-4, these cells do not differentiate into pharyngeal cells (Mango et al., 1994). The original cells that express pha-4 are multipotent; there are several different cell types they can differentiate into, such as neurons or muscle cells, but they are all pharynx cells. Similarly, the human heart has an identity gene called Nkx2-5 that is required for a cell to commit to a cardiac fate and is essential for the earliest stages of heart development (Fu et al., 1998). This is demonstrated by the fact that mutations to Nkx2-5 are directly linked to congenital heart disease (Kasahara et al., 2000). Such evidence demonstrates that it is crucial that genes regulate cell differentiation accurately in order to form functional tissues and organs in an embryo.

As cell division continues, each cell becomes isolated from its relatives, thus, in order for the genetic regulators to fulfill their role, cell to cell signaling must be activated. There are several evolutionarily conserved pathways through which these occur. These include, but are not limited to, the Notch, Wnt and Hedeghog signaling pathways, all of which are present in millions of species, both invertebrate and vertebrate (Slack, 2005). Notch signaling plays a key role throughout *C. elegans* embryogenesis.

*This author wrote the paper as a senior thesis under the direction of Dr. Pliny Smith.

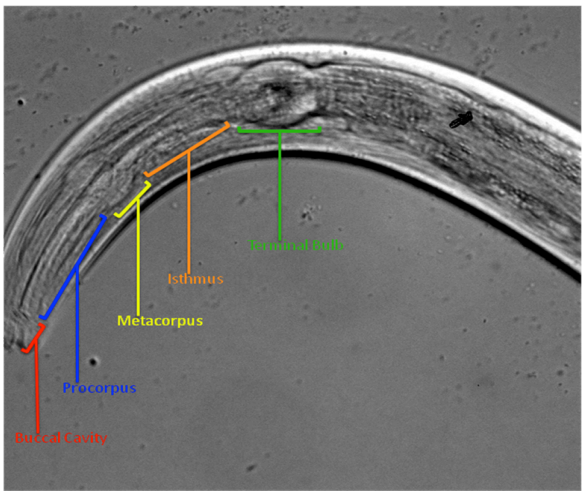

Figure 1: The *C. elegans* Pharynx: Anatomical sections of the pharynx from anterior to posterior the buccal cavity (red), procorpus (blue), metacarpus (yellow), isthmus (orange) and terminal bulb (green).

In order for cell to cell Notch signaling interactions to take place, one cell must express a receptor for a Notch ligand, while the other cell must express the ligand (Priess, 2005). For example, the precursor cell of the left side of the head of *C. elegans* expresses the Notch receptor LIN-12, while its neighboring cell expresses the Notch ligand LAG-2, the binding of which stimulates this precursor to differentiate into left head cells (Moskowitz and Rothman, 1996). When the genes encoding these signaling molecules are mutated, the left precursor is unable to undergo correct differentiation and the head does not form correctly, demonstrating the crucial role of signaling in determining cell fate, along with the use of maternal RNA and zygotic gene regulation.

Morphogenesis

The determination of cell fate is not sufficient in developing a functional organism. Once cells have committed to a particular fate, they must come together to create specialized tissues through a process called morphogenesis. Morphogenesis refers to the regulation of cell shape and movement throughout the construction of tissues and organs (Portereiko and Mango, 2001). Cells may be born and specified in one location, and must migrate to another region of the embryo where they fulfill their function. Morphogenesis is particularly important in tube formation. Tubes are essential components of several organs, namely the heart, digestive system and kidneys. In kidney tubulogenesis, for example, transcription factors, signaling pathways and adhesion molecules all play a role in the induction of mesenchymal cell migration to form tubes (Kuure et al, 2000). Mesenchymal cells are multipotent stem cells that aggregate during the formation of the kidney and undergo a transition to epithelial cells. The epithelial cells migrate and differentiate to form a long, continuous and connected epithelium, making up the tubule structures of the functional unit of the kidney, the nephron (Kispert et al., 1998). Several of the factors discussed earlier contribute to this morphogenetic process, including intercellular signaling. The signaling molecule Wnt-4 induces the formation of the continuous epithelium in the nephrons and contributes to the differentiation of the mesenchymal cells (Kispert et al., 1998). While the collaboration of numerous transcription factors and signaling molecules such as Wnt-4 contribute significantly to morphogenesis, there is another crucial element.

Cell Adhesion

The movement and placement of cells is facilitated through the presence of selected adhesion molecules. Cell adhesion has a role in cell fate and differentiation, but is particularly critical to tissue formation and morphogenesis because cells must adhere to one another, as well as to the cytoskeleton to form a three-dimensional tissue structure (Cox and Hardin, 2004; Gumbiner, 1996). Furthermore, adhesion complexes help facilitate morphogenesis in that they exert forces on neighboring cells through their connections to cause cell migration. Cells also rely on adhesion complexes such as integrins, to translate cues from the extracellular matrix (ECM) to instructions for migration (Gumbiner, 1996). Integrins are a family of transmembrane receptors that play a role in cell fate, differentiation, apoptosis and migration. Integrins bind to the ECM and transmit a signal to the interior of the cell by binding proteins of the cytoskeleton or kinases and growth factors within the cell (Giancotti and Tarone, 2003; Bokel and Brown, 2002). If a cell is not attached to the ECM through integrins, for example, it undergoes programmed cell death, which is a mechanism used by the organism for preventing cells from growing and proliferating in incorrect locations (Giancotti and Ruoslahti, 1999). The importance of adhesion complexes is illustrated in their relationship to diseases such as cancer. Integrins, in particular, are crucial to the control of the cell cycle and cell proliferation, alteration of which can lead to cancer (Giancotti and Ruoslahti, 1999; Gumbiner, 1996). Integrins can also mediate the anchorage of cancerous cells in inappropriate locations to contribute to tumor metastasis (Zetter, 1993).

Though adhesion molecules are critical to prevent disease, development is also dependent on adhesion complexes. Moreover, these molecules are essential for the construction of a three-dimensional structure, such as a complex organ.

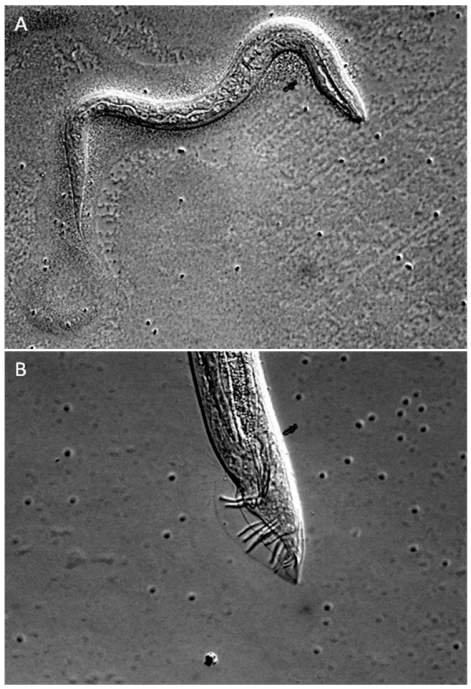

Figure 2: Hermaphrodite and Male *C. elegans*: (A) Adult hermaphrodite at 100x magnification (bright field) (B) Adult male tail at 400x magnification (bright field)

Organogenesis

Through carefully regulated processes, such as intercellular signaling and morphogenesis, functional organs develop.

Organ development is an extremely complex process and it is critical that organogenesis proceed correctly because mutant organs usually result in death of an organism. For example, the mammalian heart begins as two primitive bilateral heart tubes that must fuse together into one. The transcription factor GATA4 is required for this fusion and the lack of GATA4 at this stage results in embryonic lethality (Epstein and Buck, 2000). Following the formation of a single cardiac tube, individual chambers such as the ventricles and atria must be specified with the help of numerous genes, such as *Irx4* (Epstein and Buck, 2000). The cardiac tube must undergo a process known as looping morphogenesis in which the symmetrical tube loops to the right and develops left-right asymmetry (Breckinridge et al., 2001). In mammalian hearts, the transcription factor *Nkx2-5* contributes to the initiation of heart looping and embryos lacking this gene are also embryonic lethal (Epstein and Buck, 2000).

There are countless steps following the early development of the heart, such as valvulogenesis and the patterning of major vessels. At each point in development, there are genes and signaling molecules regulating the processes required to make a functioning heart and the interruption of the activities of these molecules can lead to congenital heart defects. For example, Holt-Oram Syndrome is a congenital birth defect characterized by arm deformities and heart defects (Mori and Bruneau, 2004). It is caused by a mutation to the gene *Tbx5*, which results in atrial or ventricular septal defects, also known as a hole in the heart (Mori and Bruneau, 2004). Moreover, these defects can cause further cardiovascular problems throughout life. Holt-Oram Syndrome is just one example of the consequences of incorrect organogenesis. It is crucial for such organs to be patterned correctly to be fully functioning and result in a normal living organism.

The Pharynx

It is a bi-lobed, linear structure consisting of six distinct sections, from anterior to posterior, the buccal cavity, procorpus, metacorpus, isthmus and terminal bulb (Albertson and Thomson, 1976; Figure 1).A basement membrane surrounds the organ and delineates it from other tissue. The pharynx consists of the following seven cell types: arcade cells, muscle, epithelial, gland, marginal cells, neurons and valve cells (Albertson and Thomson, 1976). Recently, the pharynx has emerged as an ideal model for the study of organogenesis. Because *C. elegans* is transparent, and its complete cell lineage is known, observation of pharyngeal development at every stage is very feasible (Mango, 2007). Furthermore, the anatomy of the pharynx is fairly simple and has been characterized using electron microscopy by Albertson and Thomson (1976). Another advantage possessed by the pharynx as a model for organogenesis is its ability to produce a normal pharynx when other aspects of development fail, researchers to be certain pharynx abnormalities are not aresult of indirect effects from other development issues.

The pharynx also shares several characteristics and evolutionarily conserved developmental mechanisms with the complex organs of higher-level organisms including consisting of multiple cell types originating from different cell origins using independent pathways for specification (Mango, 2007).

Genetically speaking, orthologous transcription factors have been discovered that are required for normal development of each organ. In vertebrates Nkx2-5 is a transcription factor orthologous to *C. elegans* ceh-22 transcription factor essential to the formation of pharyngeal muscle. Fascinatingly, the expression of Nkx2.5 in C.elegans rescues a ceh-22 mutant, demonstrating just how closely related the two transcription factors are (Haun et al. 1998).

C. elegans as a Model Organism

The use of model organisms is widespread throughout biology and scientific research because relevant scientific questions can be answered that would not be feasible using humans. The high occurrence of evolutionarily conserved mechanisms between vastly different animals allows for this as well. In developmental biology, there are six major model organisms, each possessing unique advantages and disadvantages. These include the mouse, chick, zebrafish, *Xenopus*, *Drosophila,* and finally, *Caenorhabditis elegans.*

C. elegans, a soil nematode approximately 1 mm in length, has been in use as a model organism since the 1960's (Mango, 2007). Its use is not limited to developmental biology, however, as it has been used as a model organism for studies in neuroscience, genetics and several other fields.

C. elegans provides several advantages for the study of many biological questions, especially in developmental biology. As mentioned earlier, its transparent body makes observation throughout embryogenesis fairly simple. Furthermore, the adult hermaphrodite possesses exactly 959 somatic cells, an invariant number, making it possible to determine its complete cell lineage (Sulston et al, 1983). *C. elegans* has a short life cycle; only 3 days from egg to adult. It is an ideal organism for genetic experiments for several reasons. It exists as both hermaphrodites and males, allowing for the crossing of different strains to obtain progeny with desired genotypes (Figure 2). An adult hermaphrodite will produce approximately 300 progeny throughout its lifetime. In addition, *C. elegans'* genome has been fully sequenced, consisting of five autosomal chromosomes and one sex chromosome (Hillier et al., 2005).

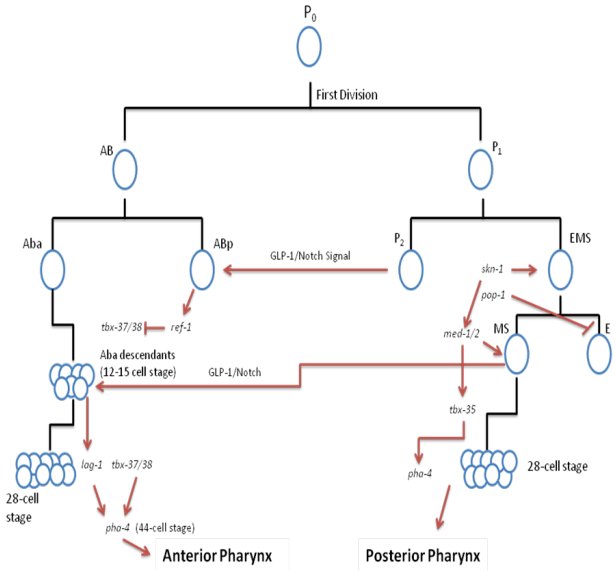

Figure 3: Cell signaling in Pharyngeal Development: Genes known to be involved in the activation of organ identity gene *pha-4* leading to commitment to anterior or posterior pharynx fate. Adapted from Ferrier (2008) using Priess (2005), Neves and Priess (2005), Good et al. (2004), Smith and Mango (2007), Mango (2005), Bowerman et al. (1992), Broitman-Maduro et al. (2006), Lin et al. (1995).

C. elegans is used as a model to study numerous diseases, for example, spinal muscular atrophy, a disease

characterized by the loss of lower motor neurons in early development. The human survival motor neuron (SMN) gene has an orthologue on
chromosome I of *C. elegans* (CeSMN), meaning there is a gene on chromosome I of *C. elegans* that possesses shared ancestry with the human gene and carries out the same function (Culetto and Sattelle, 2000). A knockout of CeSMN, in which the gene is completely removed from the organism, in *C. elegans* resulted in decreased progeny, and surviving worms that were uncoordinated and lacking muscle tonicity, symptoms similar to those observed in humans with this disease (Culetto and Sattelle, 2000). Further investigation using *C. elegans* as a model will lead to a deeper understanding of the molecular basis of the disease.

Surprisingly, *C. elegans* exhibits many mechanisms that have been evolutionarily conserved and are present in other invertebrates and vertebrates. Its genome is 40% homologous with that of humans, a result of its shared ancestry. For example, programmed cell death (PCD) is a process that occurs in nearly all organisms. In *C. elegans*, to reach the correct number of cells (959) some cells must undergo apoptosis. Interestingly, the mechanisms of PCD were found to be genetically controlled in *C. elegans* and later shown to be similar to the mechanisms governing PCD in humans (Metzstein et al., 1998). This similarity and countless others demonstrate the usefulness of *C. elegans* as a model organism in developmental biology and beyond.

Early Pharyngeal Specification

As stated before, the development of the pharynx is polyclonal, meaning pharyngeal cells originate from two different cells.

The zygote (P0) first divides to produce the AB and P1 cells. The AB cell divides along the transverse axis to produce ABa and ABp while P1 divides asymmetrically along the anterior-posterior axis to give EMS and P2 (Priess, 2005; Figure 3). Anterior pharynx cells originate from the ABa cell while posterior pharynx cells originate from the EMS cell. Both these cells, however, also produce non-pharyngeal descendants, such as body wall muscle, gonad and intestinal cells (Sulston et al. 1983). Early pharyngeal development is directed at activating the organ-selector gene: *pha-4*, a member of the FoxA transcription factor family (Mango et al., 1994). The activation of *pha-4* early in embryogenesis causes the precursor cells to commit to a pharyngeal fate. At this point, it has not yet been determined whether these cells will become neurons, muscle cells or another type of pharynx cell. Interestingly, the ABa and EMS cells exhibit different pathways to the activation of *pha-4* in their descendants.

The Anterior Pharynx

Specification of anterior pharyngeal fate begins at the 4-cell stage with maternal signaling molecules of the Notch pathway. At this point, both ABa and ABp are expressing the receptor GLP-1/Notch. However, ABp is posteriorly located and thus is in contact with the P1 descendant P2, which expresses a ligand of the Notch signaling pathway, *glp-1*, thus, Notch signaling becomes activated in ABp, but not its sister ABa (Priess, 2005, Figure 3). A key target of *glp-1* is embryonic transcription factor, *ref-1*. The *ref-1* family of transcription factors, when activated, repress the expression of two functionally redundant genes *tbx-37/38* (Neves and Priess, 2005, Figure 3). One role of *tbx-37/38* is to activate *pha-4*, the organ identity gene (Good et al. 2004). Thus, through Notch signaling, pharyngeal fate is being suppressed in the ABp cell. It then adopts secondary fates, such as an ectodermal precursor (Good et al., 2004).

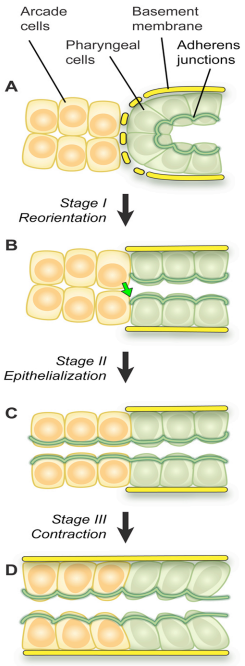

Figure 4: Pharyngeal Morphogenesis: (A) Prior to morphogenesis pharynx cells (green) are arranged as a cyst and separated from the arcade cells (yellow). (B) Anterior pharynx cells undergo "reorientation", changing their polarity to align with arcade cells. (C) "Epithelialization" results in pharynx and arcade cells forming continuous epithelium. (D) "Contraction" stage pulls pharynx anteriorly (from Mango, 2006).

Pharyngeal fate is repressed in ABp at the 4-cell stage, but it is not until the 12-15 cell stage that the second Notch signaling interaction begins the activation of *pha-4* in ABa descendants. An unknown ligand from the EMS descendant MS interacts with the GLP-1/Notch receptor that is still present on the surfaces of the ABa descendants. This induces the expression of LAG-1, which in turn activates both *pha-4* and the *ref-1* family (Smith and Mango, 2007, Figure 3). *pha-4* is also activated by *tbx-37/38* in ABa cells, but why does REF-1 not repress the expression of *tbx-37/38* in the ABa descendants as it does in ABp descendants? The timing of *ref-1* expression is crucial in this process. *tbx-37/38* is expressed at the 24-cell stage in ABa descendants, but *ref-1* is only activated at the 26-cell stage. Because *tbx-37/38* is already being expressed at this point, *ref-1* cannot repress it, allowing *tbx-37/38* to induce the expression of *pha-4* (Neves and Priess, 2005, Figure 3). *pha-4* is activated at the 44-cell stage in the ABa lineage (Mango, 2005).

The Posterior Pharynx

Contrary to the anterior pharynx development, the posterior pharynx develops from the MS cell using a Notch-independent pathway. At the 4-8 cell stage, the EMS cell is specified by maternal genes, *skn-1* and *pop-1*. *skn-1* specifies EMS descendants to become E and MS cells. The absence of *skn-1* results in EMS descendants adopting a C fate, and the complete lack of a pharynx (Bowerman et al, 1992). The C blastomere eventually differentiates into muscle tissue, hypodermis and neurons but will not produce any posterior pharynx cells (Rose and Kemphues, 1998). It is important to note that this affects the anterior pharynx as well as posterior because of the MS blastomere's role in signaling to the ABa descendants to specify a pharyngeal fate (Figure 3). *skn-1* is also responsible for the activation of *med-1,2*, transcription factors that are required to activate mesodermal identity genes that specify the MS blastomere. One of the target genes of *med-1,2* is another T-box

transcription factor, *tbx-35*. In addition to contributing to the specification of MS fate, it is thought to play a role in activating *pha-4* in the MS blastomere (Figure 3), because *tbx-35* mutants fail to produce a posterior pharynx (Broitman-Maduro et al., 2006).

pop-1 also contributes to the specification of the EMS blastomere. When cells divide along an anterior-posterior axis, *pop-1* is more abundant in the anterior sister cell and therefore specifies anterior fates (Labouesse and Mango, 1999). The EMS blastomere is one such cell that divides anterior-posterior, with the MS being the anterior cell that expresses *pop-1* (Lin et al. 1998). In this case, *pop-1* suppresses the E fate in the MS blastomere by repressing genes that promote E fate (Broitman-Maduro et al, 2006). MS descendants produce mesoderm tissue, including pharyngeal cells, while E descendants produce endoderm tissues (Sulston et al., 1983). Therefore, reduced activity of *pop-1* results in a lack of posterior pharynx cells because MS essentially adopts an E fate (Lin et al., 1995).

Pharyngeal Morphogenesis

A crucial step in the development of the pharynx is morphogenesis. The cells of the differentiated pharynx form a ball, and must elongate into a tube of eight groups of cells, set end to end and joined by adherens junctions (Albertson and Thomson, 1976). At this point, the pharynx must also be fixed to the buccal cavity at the anterior end of the worm. Pharyngeal extension begins when 78 of the 80 pharynx cells have differentiated, approximately 330 minutes after the first cell division (Portereiko and Mango, 2001).

The first stage of morphogenesis is referred to as "reorientation", in which the most anterior epithelial cells of the pharynx primordium rotate, changing their polarity, resulting in their apical surfaces, or tips, lining the future lumen of the primordium and in closer proximity with the arcade cells, the epithelial cells that will form the buccal cavity (Portereiko and Mango, 2001; Figure 4A,B). Interestingly, the anterior epithelial cells themselves have no net movement anteriorly, but are simply changing their polarity (Portereiko and Mango, 2001).

The second stage of pharyngeal morphogenesis, known as "epithelialization", is the formation of the continuous epithelium that will form the lumen of the pharynx, Prior to this, adherens junctions were exclusive to the pharyngeal cells but throughout epithelialization, adherens junctions appear in the arcade cells and anterior epidermis. Thus, the pharynx primordium, buccal cavity and anterior epidermis are connected together (Portereiko and Mango, 2001; Figure 4B,C).

The last stage of pharyngeal extension is the "contraction" stage, in which a contraction of the buccal cavity, anterior pharynx cells and epidermis pulls them together. The contraction draws the pharynx forward and the epithelial cells elongate, while simultaneously cells of the epidermis are shifting backward (Portereiko and Mango, 2001; Figure 4C,D). Interestingly, the loss of arcade cells results in essentially no contraction phase occurring, suggesting an important role for arcade cells in this process. It is possible that the arcade cells are involved in signaling events during pharyngeal extension, but this hypothesis has not been definitively supported or refuted yet (Portereiko and Mango, 2001). A common morphogenesis problem is the pharynx unattached (Pun) phenotype, basically the pharynx fails to attach to the anterior epidermis to form a connection to the mouth. *ceh-43* and *elt-5* are two genes that have loss-of-function Pun phenotypes (Aspock and Burglin, 2001; Koh et al., 2002).

At the conclusion of morphogenesis, the pharynx has transformed from a ball of differentiated cells to an elongated tubular structured that is anchored to the anterior epidermis.

Cell Adhesion in C. elegans and Its Pharynx

As discussed earlier, *C. elegans* has several evolutionarily conserved mechanisms for cell adhesion. In the pharynx the primary adhesion complex utilized are apical junctions. *C. elegans* apical junctions (CeAJ) have two domains, the apical and basal (Cox and Hardin, 2004; Figure 5). The apical domain of the CeAJ is a cadherin complex, consisting of α-catenin, β-catenin and a cadherin molecule. This cadherin complex functions to anchor actin filaments to hypodermal cells through adherens junctions. Because of this, they possess a significant role in morphogenesis, translating the force from actin bundle contraction to change cell shape and contributing to the formation of a continuous epithelium (Costa et al., 1998). Three different genes code for the cadherin complex's three proteins. *hmp-1* encodes α-catenin, *hmp-2* encodes β-catenin and *hmr-1* encodes the cadherin molecule (Costa et al., 1998). *hmp-1* and *hmp-2* mutants exhibit a "humpback" phenotype, in which bulges form on the dorsal side of the embryo during elongation. *hmr-1* mutants exhibit a "hammerhead" phenotype, characterized by the appearance of cells spilling out of the anterior portion of the worm (Costa et al., 1998). Interestingly, these proteins are present in adherens junctions throughout the entire organism, but the defects only occur dorsally, suggesting there may be other redundant proteins fulfilling the same function (Costa et al., 1998). There are other proteins, such as APR-1, that have been localized to the apical domain of the junctions, but their precise role remains unknown (McMahon et al., 2001)

The basal domain of the apical junctions is composed of two different proteins not found in the apical domain. These two proteins, DLG-1 and AJM-1 physically interact and localize to apical borders of all *C. elegans* epithelia (Koppen et al., 2001). AJM-1 is a protein recognized by the commonly used antibody MH27 that is required for epithelial integrity, but not for polarity. *ajm-1* mutants exhibit ring-shaped gaps between cells, essentially creating a separation between cells (Koppen et al., 2001). DLG-1 localizes at the apical junctions independently of AJM-1 but is required for AJM-1 localization (McMahon et al,. 2001; Koppen et al., 2001). The specific function of the DLG-1/AJM-1 complex is unknown.

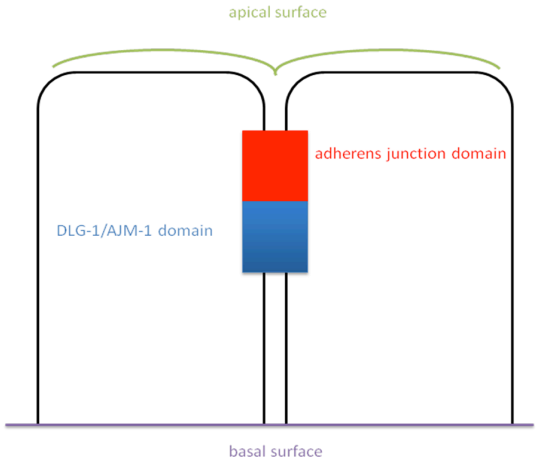

Figure 5: Apical Junction in *C. elegans*: Two cells joined by an apical junction. The adherens junction domain (red) is located apically to the DLG-1/AJM-1 domain (blue).

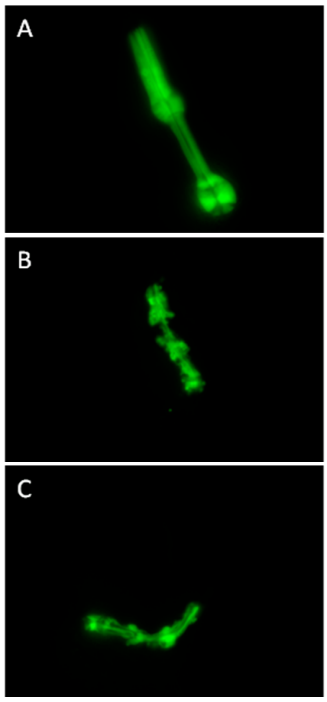

Figure 8 A-C. Mutant strains expressing *myo-2*::GFP. (A) Wildtype pharynx, compare to mutant B and C. (B) M136 mutant exhibiting severe disorganization and abnormal morphology. (C) M138 mutant showing similar disorganization and abnormal morphology in every part of the pharynx.

In the pharynx, adherens junctions form at the tip of the wedge shaped cells. Throughout morphogenesis as the marginal cells move away from the midline and the lumen expands the position of the adherens junctions changes.

Filling in the Gaps: What Additional Genes are Involved in Pharynx Development?
Though much is already known about the development of the *C. elegans* pharynx, there are copious gaps in knowledge to be filled, particularly about the process of morphogenesis and the role of the apical junction complexes. In past research, a forward mutagenesis screen produced over 200 defective pharynx phenotypes. We selected one phenotype that appeared in several strains, characterized by an extreme deformation of the pharynx causing larval lethality. We hypothesize that through the use of genetic mapping and complementation analyses to reveal the location of the gene involved, immunocytochemistry to reveal the structure of the pharynx, and fluorescent bead feeding assays to determine if the pharynx has function we will determine the identity of the gene causing this novel phenotype.

Results

Two strains, M136 and M138, with a homozygous recessive mutation were chosen from a previous mutagenesis screen for this study. While the wildtype pharynx has distinct regions, which include the procorpus, metacorpus, isthmus and terminal bulb, these regions are not discernable in M136 and M138 mutants. Both mutants exhibit pharyngeal disorganization and misshapen cells in both anterior and posterior portions of the pharynx (Figure 8). There are also gaps between pharynx muscle cells, which are not normally present. Aside from pharyngeal defects, the organism appears normal but mutant organisms arrest in the L1 stage of development so additional defects are possible.

M138 are Feeding Deficient
To investigate the larval lethality of the M138 mutant organism, we performed a functional assay to test their ability to ingest food. Wildtype worms that fed on Fluoresbrite beads for two hours exhibited beads in both their pharynx and intestine, demonstrating their ability to ingest food normally (Figure 9A,C). However, mutant organisms did not have beads present in either their pharynx or intestine (Figure 9B,D). This indicates that these are unable to feed normally thus they arrest at the L1 stage due to starvation.

Chromosomal Mapping of M136, M138 Revealed Linkage to Chromosome I
To determine the location of the mutant allele, we carried out single nucleotide polymorphism mapping. M136 mapped to chromosome I. Linkage was observed at map units -12, -6, -1 and 5 (Figure 10). The complete absence of the band representing Hawaiian DNA at map unit 5 indicates tight linkage and suggests the region of mutation in close proximity to map unit 5. At this location, the wild-type lane contains the 494 bp band of the Bristol strain and the 365 and 129 bp bands of Hawaiian DNA. The mutant lane, however, is missing the 365 and 129 bp bands of Hawaiian DNA.

The Chromosomal Map of M138 Also Demonstrates Linkage to Chromosome I.
Once again, tight linkage is seen at map unit 5, at which the mutant DNA lane lacks the 365 bp band of Hawaiian DNA (Figure 11). Although DNA is not present in several lanes that represent areas on chromosome I, there is no linkage seen in lanes representing any other chromosomes, thus we can conclude the mutation is linked to chromosome I.

Interval Mapping of M136
To narrow down the location of the mutant allele, the second phase of SNP mapping, interval mapping, was carried out on the M136 strain. The interval mapping procedure was similar to that of chromosomal mapping. However, interval mapping examines the individual genotypes at a specific chromosomal location, rather than the genotypes of a group of organisms.

The four chromosomal loci we chose to interval map for M136 were those that exhibited linkage to the middle of chromosome I. The interval map of map unit 5 contained only one lane of recombination, in which both the Bristol band at 494 and the Hawaiian band at 365 were seen (Figure 12B). Six lanes did not contain any bands. The recombination frequency at map unit 5 was calculated to be 0.56%, suggesting that the mutation is located between 5 + 0.56 and 5 – 0.56 (Table 3).

The interval map of map unit -1 exhibited sixteen areas of recombination and only three lanes completely lacking DNA (Figure 12A). The recombination frequency was calculated to be 8.6% (Table 3), thus we inferred the mutation is located between map units -1 + 8.6 and -1 – 8.6, which agrees with the range determined by map unit 5. Map unit 13 resulted in a recombination frequency of 5% (Table 3). Eight areas of recombination were observed and sixteen lanes did not contain any DNA (Figure 12C). The resulting range was between 13 + 5 and 13 – 5 map units. Map unit 13 resulted in a recombination frequency of 5% (Table 3). Eight areas of recombination were observed and sixteen lanes did not contain any DNA (Figure 12C). The resulting range was between 13 + 5 and 13 – 5 map units.

Table 3: Recombination Frequencies from Interval Mapping of M136

Interval	Lanes with Recombination	Total Chromosomes	Recombination Frequency
-1	16	186	8.6%
5	1	180	0.56%
13	8	160	5.0%
14	23	176	13%

*Recombination frequency was calculated by $\frac{\text{areas of recombination}}{\text{total chromosomes}} \times 100$

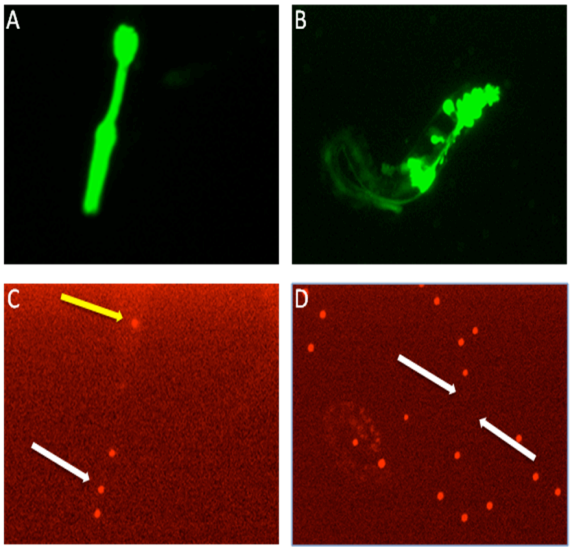

Figure 9 A-D. Feeding Assay of M136. (A) WT pharynx expressing *myo-2*::GFP. (B) M136 mutant pharynx expressing *myo-2*::GFP. (C) Fluoresbrite beads are present in pharynx (white arrow) and intestine (yellow arrow). (D) No Fluoresbrite beads are present in mutant pharynx

Thus, after three interval maps, the range of mutation is roughly between map units 5 and 8. However, the interval map of map unit 14 exhibited twenty-three areas of recombination with a resulting recombination frequency of 13%, significantly higher than expected (Figure 12D; Table 3). Therefore, interval mapping revealed the approximate range containing the mutant allele to be between map units 1 and 8 on chromosome I.

Complementation Analysis: M136 and M138 Mutant Phenotypes are Caused by an Allele on the Same Gene

The results of the chromosomal mapping of M136 and M138 showed both strains to map to the same approximate area on chromosome I. These strains also exhibit similar phenotypes with extensive disorganization and abnormal morphology of cells. This evidence suggests that M136 and M138 mutant phenotypes may in fact be caused by a mutation on the same gene.

To determine whether or not this is the case, we performed a complementation analysis. The M136 and M138 strains are homozygous recessive, therefore to exhibit a mutant phenotype when they are crossed, they must still have two copies of the mutant allele (Figure 13). If different genes cause the mutations to the different strains, complementation will occur and no mutant organisms will be observed in the offspring. However, if the mutations are caused by alleles on the same gene, approximately 25% of the offspring will exhibit the mutant phenotype of that gene.

Our results showed approximately 20% of the offspring demonstrating the mutant phenotype. Therefore, the mutant phenotypes of M136 and M138 are caused by a mutation to the same gene. However, they may still be caused by different alleles *Antibody Stain of Intermediate Filaments Reveals Marginal Cell Defects.* M136 and M138 mutants exhibit severe disorganization of the pharynx and appear to have misshapen cells. However, little information can be discerned about what cells are affected and in what way by just looking at the myo-2::GFP images. To investigate more thoroughly which pharynx cells are being affected by this mutant allele, an antibody stain of the intermediate filaments was performed. The intermediate filaments are the main component of the marginal cells in the pharynx. There are three sets of three marginal cells in the pharynx running longitudinally from the buccal cavity to the terminal bulb totaling nine cells (Albertson and Thomson, 1976; Figure 14A,B).

Genetic Location Primers Genetic Location Primers

The antibody stain of M136 revealed marginal cell defects. Wild-type marginal cells appear long, smooth and uniformly shaped from the buccal cavity to the terminal bulb (Figure 14E). All wildtype embryos viewed and photographed exhibited this pattern in the marginal cells (Table 4). The mutant embryos did possess the intermediate filaments of the marginal cells, however, they did not display wild-type characteristics. The marginal cells were disjointed instead of smooth, and there were portions that did not stain, suggesting intermediate filaments may be lacking in this area (Figure 14F). One hundred percent of mutant embryos demonstrated marginal cell defects, though the disorganization varied slightly from embryo to embryo (Table 4).

Antibody Stain of the Adherens Junctions

The pharyngeal muscle tissue of the M136 and M138 mutant organisms is severely malformed. This is possibly due to a lack of adhesion of individual muscle cells resulting in the misshapen cells and abnormal spaces between cells (Figure 8B,C). To investigate this, we used the monoclonal antibody MH27 to determine whether or not adherens junctions were present in the mutant organisms. The adherens junctions of the pharynx are clearly discernible from those in the hypodermis and other areas because of increased staining intensity at the buccal cavity, metacorpus and terminal bulb in the wildtype worms (Figure 15A). Of eleven wildtype embryos viewed and photographed, all demonstrated this pattern of adherens junction staining (Table 5). Anti-GFP antibody was used with the intention of staining the pharynx

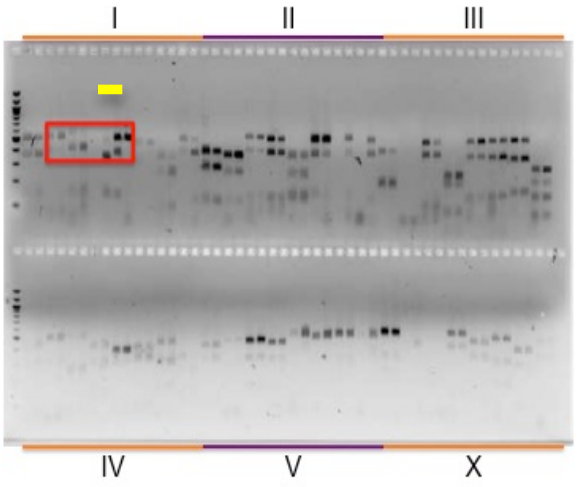

Figure 10: Chromosome Map of M136. Agarose gel electrophoresis of PCR reactions in chromosome mapping. Roman numerals represent each chromosome and each SNP region is represented by a pair of lanes, 8 SNP regions per chromosome. In each pair, the first lane represents wild-type DNA, the second lane represents mutant DNA. The red box illustrates the area of the mutant allele. Note the missing band in the region highlighted by the yellow line, suggesting tight linkage is this area.

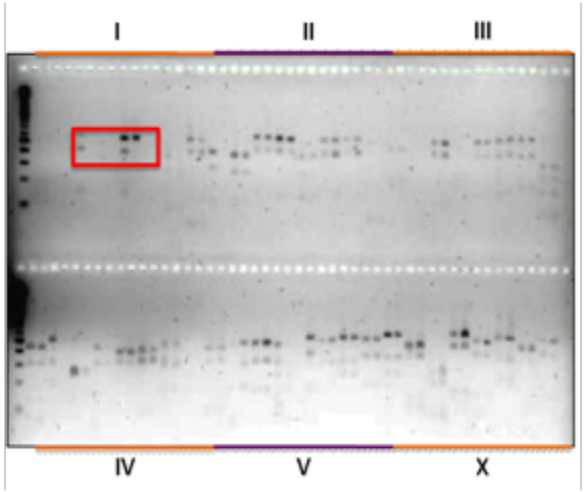

Figure 11: Chromosome Map of M138. Agarose gel electrophoresis of PCR reactions in chromosome mapping. Roman numerals represent each chromosome and each SNP region is represented by a pair of lanes, 8 SNP regions per chromosome. In each pair, the first lane represents wild-type DNA, the second lane represents mutant DNA. The red box illustrates the area of the mutant allele. Note the missing band in the region highlighted by the yellow line, suggesting tight linkage is this area.

asmyo-2::GFP is destroyed through the staining process. However, the anti-GFP stained indiscriminately so it is not possible to distinguish the pharynx (Figure 15B).
In spite of this, the embryo could clearly be classified as wildtype because the pharynx is clearly visible in the DIC image (Figure 15C). The possible mutant embryo did not show any definitive pharyngeal adherens junction staining but the adherens junctions of the hypodermis are more clearly visible (Figure 15D). There is a higher intensity of color in the center of the mutant embryo that could possibly be a pharynx but there is no distinct, elongated structure as in the wildtype embryo Because the anti-GFP stain was not consistent, this embryo cannot be definitively classified as mutant (Figure 15E). No embryos were seen, though, that had clear pharyngeal adherens junction staining that were not categorized as wildtype using a DIC image.

Genetic Cross of SM1493 with M136
The mutant worms of the M136 and M138 strains appear to have gaps between muscle cells that are not normally present, which could be the result of irregular adherens junctions causing the cells to remain detached or some muscle cells may not be present at all. To determine if all muscle cells were present in the mutant worms, we carried out a genetic cross between SM1493 and M136 to create a strain that expressed the mutant allele, but also possessed muscle cell nuclei that expressed GFP (Figure 16A-D). Compared to the organized and consistent wildtype pharynx, the nuclei appeared in random locations throughout the mutant pharynx (Figure 16A,C). By counting the number of nuclei visible in wildtype and mutant pharynges, we demostrated that there is no significant difference between the numbers of muscle cells seen in the two phenotypes (Figure 16E). The mean of the wildtype count was 22 and the standard deviation was 5.15 while the mean of the mutant count was 18.6 and the standard deviation was 3.76 and both phenotypes had a median of 20 (Figure 16E). The t-test value was 0.227 and resulted in a p-value of 0.823, which confirmed the differences in the two counts were not significant.

Discussion

The goal of our lab is to identify genes causing mutant phenotypes in the *C. elegans* pharynx with the hopes of assembling a pathway for normal pharynx development. In this study we have characterized two homozygous mutant strains, M136 and M138, as exhibiting extreme disorganization of the pharynx. Our goal was to determine the location and identity of the alleles responsible for these mutant phenotypes. We hypothesized the defects were caused by abnormal morphology and pharyngeal cell adhesion. Cell adhesion is critical to morphogenesis, an important developmental step that refers to the regulation of cell shape and movement especially during tissue and organ formation. In addition to this, adhesion complexes are responsible for the adherence of one cell to another. In this project, we used genetic, molecular and histological techniques to characterize the mutant strains.

M138 are Feeding Deficient Resulting in Larval Lethality
The inability of M138 mutant organisms to ingest food indicates that their larval lethality could be caused by starvation. Though this was assumed to be true prior to the feeding assay because of the abnormal muscle morphology, it was necessary to demonstrate their inability to eat for starvation to be considered a possible cause of death. However, because the ethylmethanesulfonate (EMS) used in the mutagenesis screen from which the M136 and M138 strains were created causes mutations to occur in multiple genes in each strain, it is possible a different mutation is causing larval lethality (Epstein and Shakes, 1995). Because M136 and M138 have been backcrossed with the Hawaiian strain, which helps eliminate incidental mutations, and heterozygous hermaphrodites produce mutants at a 1:4 ratio, it is unlikely another mutation is causing the lethality.

M136 and M138 Mutant Alleles are Located on Chromosome I Between Map Units 1 and 8
Through the use of single nucleotide polymorphism mapping we determined the mutant alleles of M136 and M138 to be located on Chromosome I. Interval mapping further narrowed down the region of DNA to between map units 1 and 8.

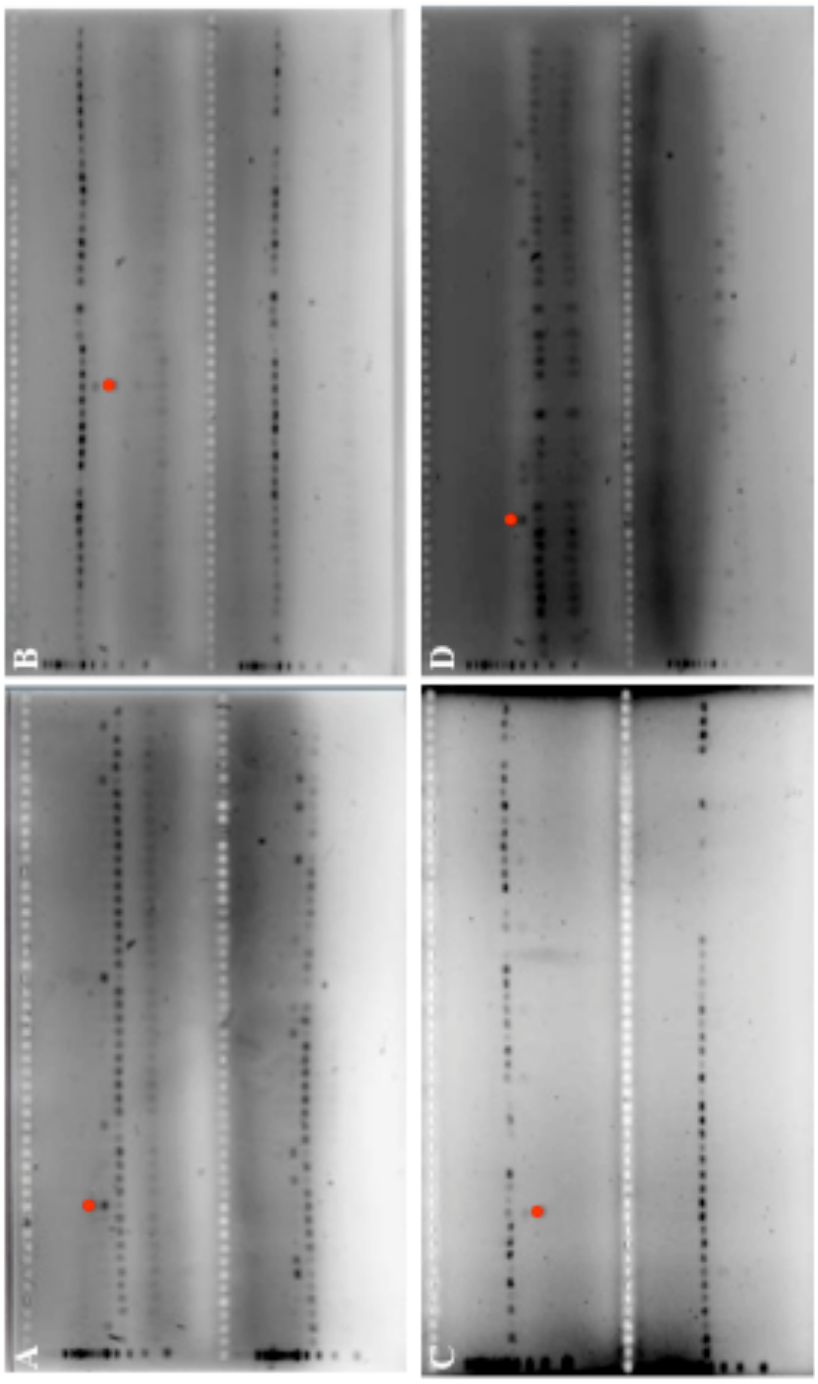

Figure 12. Interval mapping of M136. (A-D) Agarose gel electrophoresis of interval maps. Each lane represents one mutant genotype. One recombinant lane for each gel is marked with a red circle as an example. Lanes with an additional band of DNA indicate recombination has occurred in this organism. (A) Interval map of map unit -1 on chromosome I showing 16 areas of recombination (B) Map unit 5 of chromosome I exhibits one area of recombination (C) Map unit 13 on chromosome I shows eight areas of recombination. (D) Map unit 14 on chromosome I shows 23 areas of recombination

Using the *C. elegans* database Wormbase, we searched for genes within this region that demonstrated similar pharynx phenotypes to our mutant strains. *hmr-1* is located at map unit 6.63 on chromosome I (Wormbase website). This gene codes for one of the three proteins in the cadherin complex that makes up the apical domain of *C. elegans* apical junctions (Costa et al., 1998). The cadherin complex plays a crucial role in morphogenesis of the pharynx. The cadherin complex fixes the actin filaments to the hypodermal (outermost) cells allowing for the contraction to shift the hypodermal cells and facilitate elongation (Priess and Hirsh, 1986).

A pharynx phenotype has not yet been classified for *hmr-1*, and its role in morphogenesis makes it a plausible candidate for the gene involved in the mutant phenotypes of our M136 and M138 strains. Conversely, *hmr-1* displays a "hammerhead" phenotype in which cells appear to be spilling out of the anterior end of the organism (Costa et al., 1998). This phenotype is not seen in M136 and M138, indicating that *hmr-1* is not the mutant allele contributing to our mutant strains. *hmp-1* and *hmp-2* are additional genes involved in adherens junctions in the *C. elegans* pharynx but are located on chromosome V and on chromosome I at approximately map unit 26, respectively, neither of which correlates with the location of our mutant gene (Wormbase). Moreover, these two genes exhibit similar pharynx phenotypes, but additional body defects not seen in our mutants (Costa et al., 1998). As of yet, our phenotypes seem to be unique, suggesting that if this gene is known, its effect on the pharynx is unknown, or the gene has yet to be classified at all. However, to draw any more significant conclusions, the map unit range within which the mutant allele is located must be further narrowed down and the exact sequence determined.

M136 and M138 Mutant Phenotypes are Caused by a Mutation to the Same Gene

Both M136 and M138 localized to chromosome I as determined by SNP mapping. M136 was further mapped to between map units 1 and 8. These strains display very similar phenotypes, and their localization to the same chromosome led us to hypothesize their phenotypes could be caused by mutations to the same gene. The results of our subsequent complementation analysis supported this, making it redundant to interval map M138 as well as M136. Thus the mutant allele causing the M138 phenotype can be assumed to be located between map units 1 and 8 as well. Because M136 and M138 are located on the same gene, it does not indicate their mutations are identical as they may be a result of different mutant alleles.

By confirming that M136 and M138 are caused by mutations to the same gene, the assumption that there is only one mutant gene causing the phenotype is supported. During the mutagenesis screen from which these strains were created, there were 5000 hermaphrodites exposed to the EMS mutagen, which causes approximately eighteen heterozygous mutations per organism (Epstein and Shakes, 1995). There is a 50% chance the offspring will have the mutation in homozygous form, resulting in about nine mutations per organisms. By multiplying the number of worms with the number of mutations per worm, it can be estimated that 45 000 genes could receive a mutation. Since the *C. elegans* genome consists of approximately 19 000 genes, there is approximately a one in 19 000 chance that the same gene could be mutated in two different organisms, which is quite possible if there are approximately 45 000 mutations that will occur (*C. elegans* Sequencing Consortium, 1998). Furthermore, it is very unlikely that the same two genes were mutated in two different organisms, because the odds of that occurring are one in 361 million (190002). Therefore, it is extremely doubtful that the mutant genes, there is approximately a one in 19 000 chance that the same gene could be mutated in two different organisms, which is quite possible if there are approximately 45 000 mutations that will occur (*C. elegans* Sequencing Consortium, 1998). Furthermore, it is very unlikely that the same two genes were mutated in two different organisms,because the odds of that occurring are one in 361 million (190002). Therefore, it is extremely doubtful that the mutant phenotypes of M136 and M138 are caused by mutations to two or more genes.

There is a strong possibility that M136 and M138 actually have the same mutation for several reasons. The mutagenesis screen used to create these strains mutates the germ cells in wildtype hermaphrodites. If the hermaphrodite that produced these two mutants was at the L4 stage or younger during the application of the EMS, its germ cells may still have been dividing mitotically (Epstein and Shakes, 1995). This would allow the nucleus of the cell, including its altered genetic information, to be cloned therefore this hermaphrodite would have more than one identical progeny carrying the mutation (Epstein and Shakes, 1995). The M136 and M138 strains could be a result of a situation like this, especially since the strain numbers are so close. Moreover, they exhibit such similarity in their phenotypes, making it extremely likely that they are caused by the same mutant allele. To determine if this is the case, the mutant genomes would need to be sequenced and compared to discover any discrepancies. Antibody Stain of Intermediate Filaments Reveals Marginal Cell Defects. In the M136 mutant organisms, the marginal cells are clearly present, but staining is inconsistent indicating that portions of the marginal cells are lacking filaments.

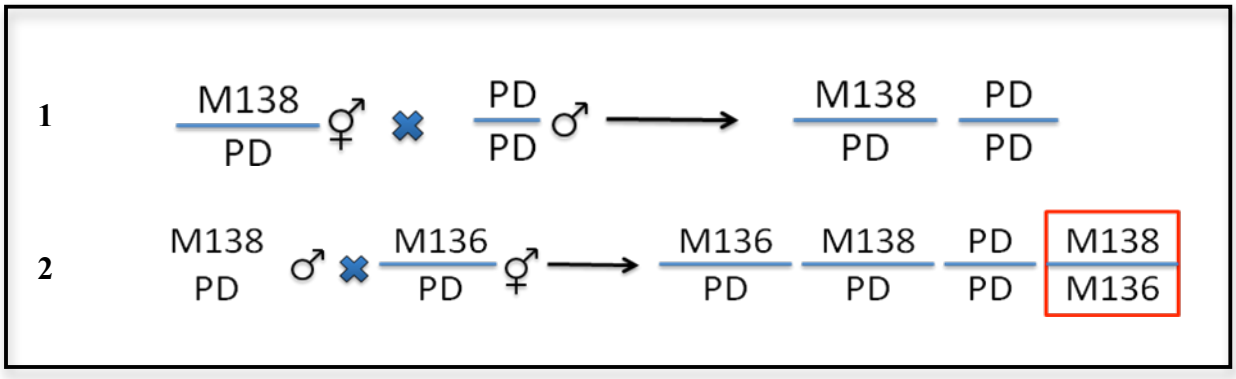

Figure 13: Complementation Schematic. PD represents the original wild-type strain PD4972 with *myo-2*::GFP. M138 heterozygous hermaphrodites were mated with wild-type males. F1 males were mated with M136 heterozygous hermaphrodites resulting in four possible genotypes. Red box highlights genotype that is either homozygous recessive or not depending on whether or not the strains complement.

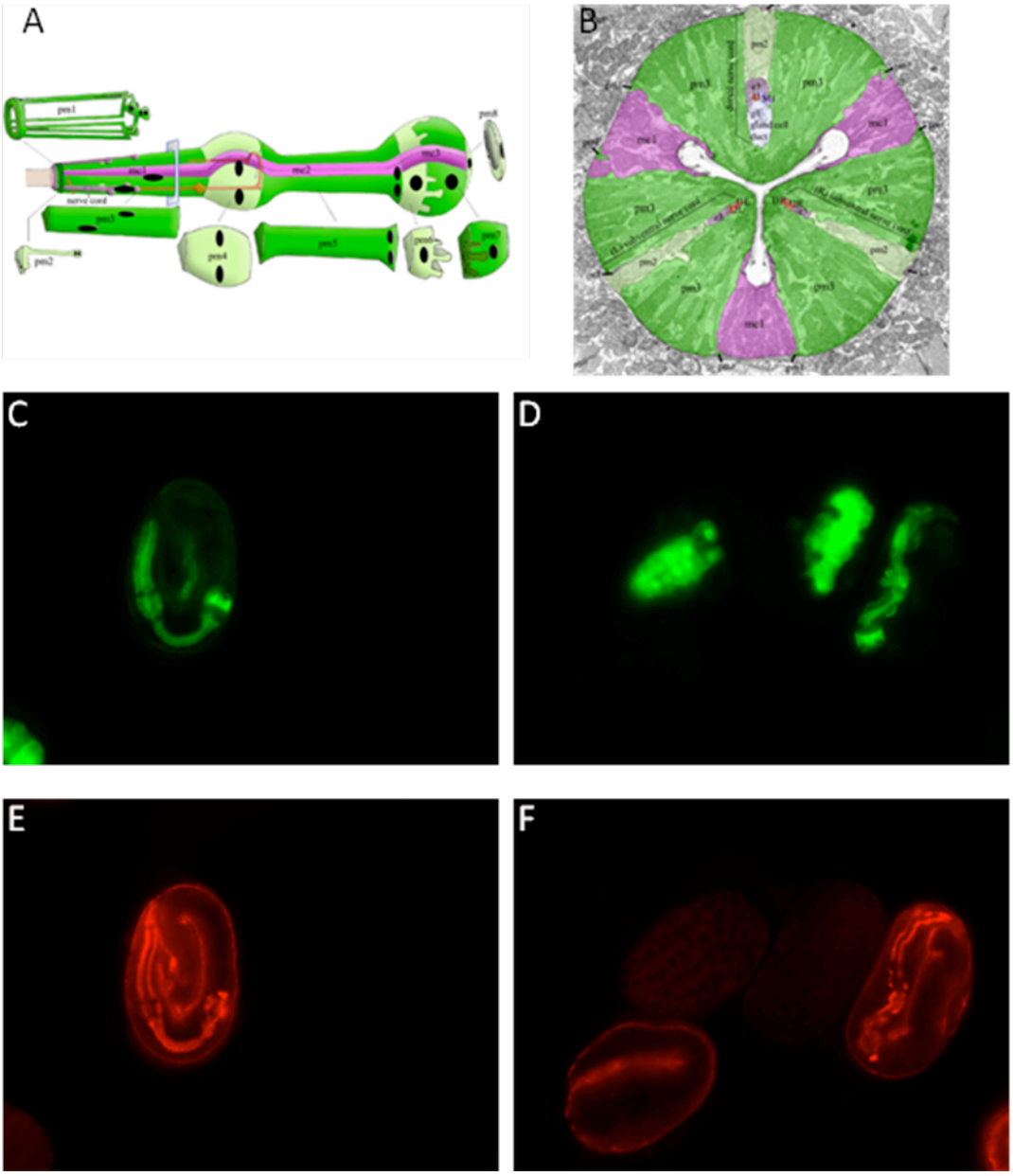

Figure 14. Antibody Stain of Intermediate Filaments. (A,B) from Mango, 2006. (A) Pharynx diagram with three marginal cells from buccal cavity to terminal bulb shown in purple. (B) Cross-section of pharynx showing three sets of marginal cells in purple. (C) GFP staining of wild-type embryo. (D) GFP staining of mutant embryo. E) Intermediate filament staining of wild-type embryo. (F) Intermediate filament staining of mutant embryo.

Because the marginal cells appear disjointed in mutants as opposed to the long uniform shape of the wildtype marginal cells, the intermediate filaments are abnormally shaped or positioned incorrectly. Intermediate filaments are an essential element of the cytoskeleton, in collaboration with actin filaments and microtubules (Goldman, 2001). The cytoskeleton's primary function is upholding the structure of the cell, therefore it is logical that the intermediate filaments in the marginal cells function to maintain the integrity of the pharynx, though the exact function of marginal cells is still under debate (Albertson and Thomson, 1976). However, it is doubtful that the irregularities of the marginal cells in mutant organisms are due to a mutation in the intermediate filament genes for the following reasons. There are eleven genes in the *C. elegans* genome coding for the intermediate filament proteins, five of which cause abnormal phenotypes (Karabinos et al., 2001). The primary intermediate filament expressed in the pharynx marginal cells is IFB-1, but this expression is not limited to the pharynx. It is also largely expressed in the epidermis at the attachment sites to the body wall muscle (Woo et al., 2003). IFB-1 mutants exhibit abnormal elongation, the last stage of embryogenesis in which the bean-shaped embryo becomes a long thin worm,

Table 4: Number of Embryos in Intermediate Filament Antibody Stain

Number of WT Embryos Viewed	Number of WT With Same Staining Pattern	Number of Mutant Embryos Viewed	Number with Abnormal Intermediate Filament Staining
4	4	4	4

Table 5: Number of Embryos in Adherens Junction Antibody Stain

Number of WT Embryos Viewed	Number of WT with Same Staining Pattern	Number of Possible Mutant Embryos Viewed	Number with no Definition of Pharynx Adherens Junctions	Number with Slight Definition of Pharynx Adherens Junctions
11	11	8	6	2

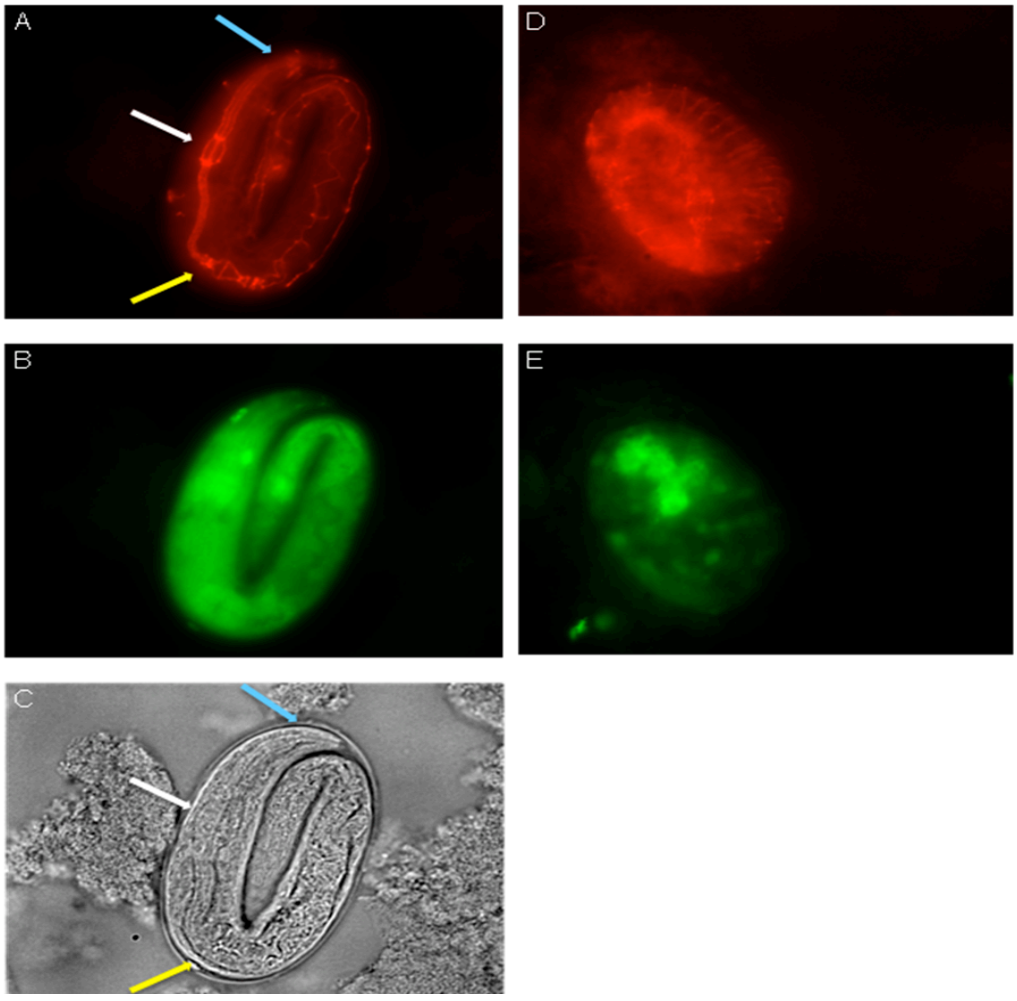

Figure 15. Antibody Stain of the Adherens Junctions (A) Wildtype embryo stained with MH27 antibody. Concentration of stain at the buccal cavity (blue arrow), metacorpus (white arrow) and terminal bulb (yellow arrow). (B) anti-GFP failed to stain only the pharynx as was intended. (C) Wildtype pharynx is evident in DIC image with buccal cavity (blue arrow), metacorpus (white arrow) and terminal bulb (yellow arrow). (D) Possible mutant embryo stained with MH27 with no distinguished pharynx but other adherens junctions exhibit staining. (E) Anti-GFP may have recognized pharynx but image is not definitive.

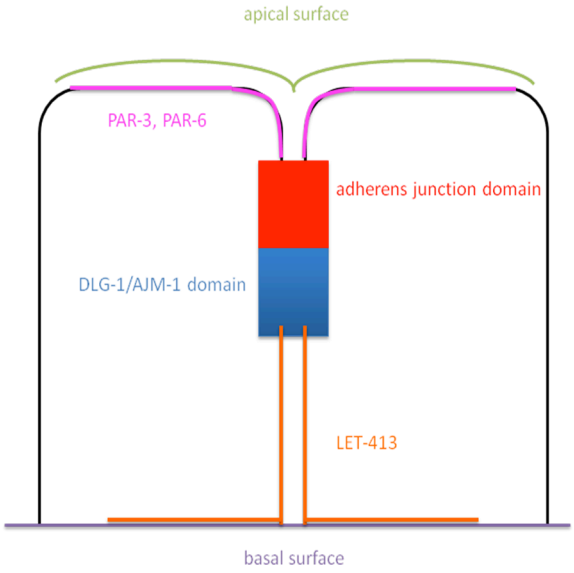

Figure 17. Representation of the *C. elegans* apical junction. PAR-3 and PAR-4 (pink) localize to the apical surface of the cells. The adherens junction domain (red) is apical to the DLG-1/AJM-1 domain (blue)within the junction. LET-413 (orange) localizes to the basolateral surfaces of the cell.

M136 Mutant Organisms Exhibit Abnormal Pharyngeal Adherens Junctions

The antibody MH27 recognizes the AJM-1 protein that is present in the basal domain of the *C. elegans* apical junction along with DLG-1 (McMahan et al., 2001). The basal domain of the apical junctions that are present in the pharynx are known to regulate adhesion (Cox and Hardin, 2004). The lack of consistent staining in mutant M136 embryos supports our hypothesis that a disruption to the adherence of one cell to another is contributing to the mutant phenotype. AJM-1 localizes to all *C. elegans* epithelia on the apical side in tissues including the hypodermis, pharynx, intestine, vulva and uterus (Koppen et al., 2001). ajm-1 mutants arrest as embryos because of an inability to undergo the elongation phase of development (Koppen et al., 2001). Though our mutants appear to lack the AJM-1 protein in their pharyngeal adherens junctions, a mutation to the ajm-1 gene cannot be the cause of this phenotype. M136 and M138 mutants are larval lethal, not embryonic, and exhibit adherens junction staining in other areas of the embryo, suggesting AJM-1 is present in these other locations. There are two other key proteins required for AJM-1 to assume its correct location in apical junctions. DLG-1 is necessary for AJM-1 to localize subapically, and LET-413, from the family of LAP and PDZ proteins, is required to position all *C. elegans* apical junction proteins, including *HMP-1* (McMahon et al., 2001; Figure 17). The absence of DLG-1 resulted in discontinuous staining using MH27, a pattern that was consistent throughout the embryo, unlike M136 mutants that appear to have normal adherens junctions staining in the epidermis but not in the pharynx. LET-413 has a significant role in the regulation of the polarity of the epithelial cells, and the absence of LET-413 results in the proteins PAR-3 and PAR-6, normally localized apically, to move to more basal locations (McMahon et al., 2001). Because our embryos only exhibit pharyngeal adherens junction defects, it is unlikely the gene being affected is let-413. It is likely that there is some redundancy in the proteins involved in adhesion complexes in *C. elegans*. This hypothesis is supported by the *hmp-1*, *hmp-2* and *hmr-1* mutants that demonstrate phenotypes with adhesion defects, but not throughout the entire worm (Costa et al., 1998). This suggests that there must be other proteins that are sufficient to maintain the adhesion complexes in the absence of other vital proteins. Since M136 and M138 appear to only suffer adhesion defects in the pharynx, it is likely the mutation to a gene crucial for pharynx adherens junctions but redundant in those in the hypodermis and other tissues is causing our phenotype.

M136 Mutants Are Not Lacking Pharynx Muscle Cells

Because the complete cell lineage of *C. elegans* is known, it is known that the pharynx is made up of exactly twenty muscle cells with 37 nuclei as a result of the fusion of several cells during development (Albertson and Thomson, 1976). The use of myo-2::GFP::H2B allowed us to determine if the M136 mutant worms possessed the correct number of pharynx muscle cells by counting the muscle cell nuclei in both wildtype and mutant worms. Though wildtype worms were expected to show exactly 37 pharynx muscle cell nuclei, there were less seen in every wildtype worm observed for the following reasons. The exposure level used prevented each nucleus from being differentiated accurately because they may have been positioned atop one another producing a very intense signal that could represent one or more nuclei. Also, some may not have been seen at all because the exposure level was too low for them to be discernable. Although not all muscle cell nuclei could be distinguished, there was no significant difference between the number seen in the wildtype pharynx compared to the mutant pharynx, indicating that the mutant worms are not lacking any muscle cells. This suggests that the abnormal gaps between muscle cells in the mutant pharynx are not caused by missing cells because they all appear to be present. This supports our hypothesis that the mutant phenotype is caused by the abnormal morphology and adhesion of pharynx muscle cells. The random positioning of the muscle cell nuclei in the mutant pharynx as compared to the wildtype pharynx also supports the hypothesis that the muscle cells do not have the correct morphology.

Limitations and Criticisms

There are a number of areas for improvement within this project. In the interval mapping experiments, several lanes often showed no DNA at all, which affects the accuracy of the recombination frequencies calculated from these maps. The interval maps of M136 could have been repeated several times to compensate for experimental error to obtain more accurate recombination frequencies. This would allow us to narrow down the range of map units within which our mutation must lie. Also, while both antibody stains exhibited differences between mutant and wildtype, more embryos must be viewed and the number demonstrating the same phenotypes must be counted to determine how often the defects are occurring to give our data more significance.

Lastly, the muscle cell nuclei counting experiment could improve if better equipment was available to visualize all 37 muscle cell nuclei within the pharynx, which would allow us to be completely sure there is no difference in the number of cells seen in the mutant compared to the wildtype.

Summary

The goal of this project was to determine the identity of the mutant allele causing the phenotypes seen in the M136 and M138 strains. We hypothesized this phenotype was a result of abnormal morphology and cell adhesion. We localized the mutant allele to chromosome I, between map units 1 and 8, within which no known gene appears to exhibit our phenotype. We have also shown that while the correct

number of muscle cells are present, the mutation affects the marginal cells and the adherens junctions as well as muscle cells within the pharynx. Future studies will include finding the exact sequence of the mutant allele through complementation analyses with deletion strains, enabling us to perform a transgenic rescue and positively identify the gene causing our unique phenotype.

Materials and Methods

Media Protocol
Nematode Growth Medium (NGM) plates were prepared in the following manner: 3.0 g NaCl, 2.5 g peptone, 17 g Bacto-agar and 975 mL water to 2000 mL Erlenmeyer flask. Autoclaved for 50 minutes, allowed 15 minutes to cool. While stirring, added 1 mL 1 M $MgSO_4$, 1 mL 1 M $CaCl_2$, 1 mL 10mg/mL cholesterol in ethanol and 25 mL $KHPO_4$ of pH 6.0. Plates were poured with a Tritech Research III in 10.0 mL volumes, and seeded with 200 μL of OP50 *E. coli.*

C. elegans Growth and Maintenance
C. elegans strains were obtained from the *Caenorhabditis* Genetics Center (CGC). The strains used in this work were PD4792 (mIs11 IV), Hawaiian CB4856 and SM1493. Mutant strains were obtained from frozen stock of previous mutagenesis screen. Worms were maintained on 6 cm NGM plates with 120 μL of OP50 *E. coli* at temperatures of 12°C, 15°C, 22°C and 25°C. Worm transfer was carried out with a 2.5 cm section of platinum wire fixed in a Pasteur pipet.OP50 *E.coli* was added to the pick to transfer the worms from plate to plate. Mutant strains were maintained by picking five to eight phenotypically wild-type (WT) hermaphrodite offspring from a known heterozygous hermaphrodite to their own plates every five days and screening the progeny for mutants. If the hermaphrodite produced mutant offspring that plate was classified as mutant.

Feeding Assay
Fluoresbrite Polychromatic red 0.5 micron Microspheres (beads) were diluted to 1:100 ratio with nanopure water and 200 μL of the mixture was added to a plate already seeded with 200 μL of OP50 *E. E. coli*. The plate was rubbed gently to mix the *E. coli* with the Fluoresbrite beads. Ten M138 wildtype L1 worms and ten M138 mutant L1 worms were picked to the plate and allowed two hours to feed. After allotted time the worms were transferred to a 4% agarose pad on a slide and covered. The slide was viewed under a Zeiss Axiovert 100 microscope and photographed.

SNP Mapping
Single nucleotide polymorphism mapping uses 48 of the SNPs between the Hawaiian and Bristol strains of *C. elegans* as genetic markers to determine the location of a mutant allele. SNP mapping is carried out in two phases; chromosome mapping, which determines the particular chromosome that carries the mutant allele, and interval mapping, which narrows down the specific location of the mutation to within a few map units.

Chromosomal Mapping
The chromosomal mapping procedure was adapted from Davis et a. (2005). In summary, ten male Hawaiian CB4856 worms were crossed with 10 heterozygous N2 hermaphrodites of the chosen mutant strain. After 24 hours, each hermaphrodite was removed to its own plate bearing a copulatory plug as evidence mating had occurred. The F1 generation was screened for males to ensure the cross had taken place. The F2 generation was screened for mutant phenotypes. Fifty mutant worms and fifty wild-type worms were picked into separate 0.2 mL tubes containing 20 μL of worm lysis buffer (60 μg/mL Proteinase K, 50μM KCl, 10μM Tris, 25μM $HgCl_2$, 0.45% Igepal, 0.45% Tween20, 0.01% gelatin). The worms were frozen at -80° C for twenty minutes then incubated at 65° C for one hour and 95°C for fifteen minutes.

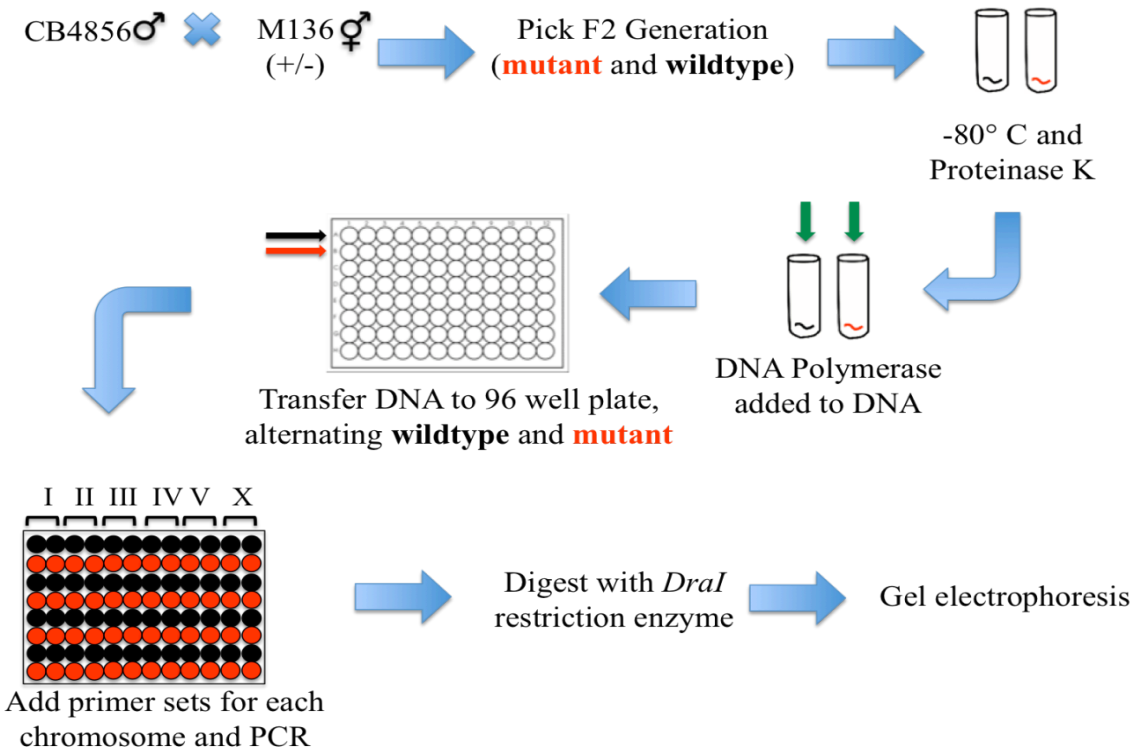

Figure 6: SNP Mapping Procedure. Schematic of the necessary steps in the SNP mapping protocol

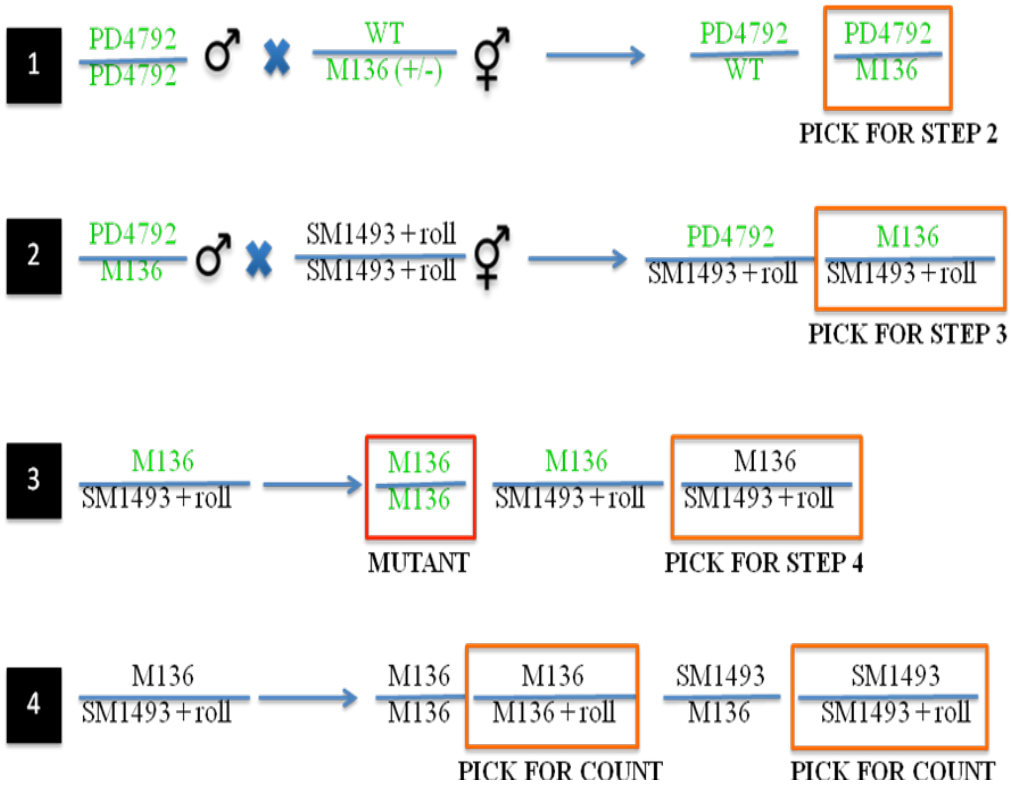

Figure 7: Genetic Cross of SM1493 and M136. Schematic showing the matings carried out to obtain mutant worms exhibiting *myo-2*::GFP::H2B. Green indicates worms of this genotype expressed *myo-2*::GFP. Genotypes including roll have the *myo-2*::GFP::H2B as the traits are linked on an extrachromosomal array and not part of the genome.

The worm lysate DNA was then added to separate 1.5 mL microfuge tubes (mutant and wild-type). 510 μL NEB 2X Taq MasterMix DNA polymerase, 40 μL $MgCl_2$ and 470 μL nanopure H_20 were added to each tube. The mixtures were distributed in 10.0 μL volumes to alternating rows (WT and mutant) of a 96-well PCR plate using a repeat pipetter. 2.5 μL of a primer set (100μM) were added to each well. Each primer pair contained forward and reverse primers representing the location of one of the 48 SNPs, eight per each of the six chromosomes (Table 1).

electrophoresis. 6 μL of Orange G loading dye was added to each well and 16 μL of sample from each well were loaded onto a 2.5% agarose gel. 100 bp ladder was loaded into the first well of each row. The gel exhibited each of the 48 SNP markers, with lanes in pairs of WT and mutant DNA. All bands for Hawaiian and Bristol DNA are known (Table 2). The DNA was analyzed by comparing wildtype and mutant DNA bands and locating discrepancies as linkage sites.

Interval Mapping

Interval mapping was adapted from Davis et al. (2005). In summary, we crossed 10 male Hawaiian strain worms with 10 hermaphrodite N2 organisms of the chosen strain. After 24 hours, each hermaphrodite was removed to its own plate. The plates were screened after 3 days for male offspring to ensure the mating was successful. 96 F2 generation mutants were picked and placed into one well of the PCR tubes containing 5 μL of worm lysis buffer each. The worms were placed in the -80°C freezer for twenty minutes then removed to the thermocycler and incubated at 65°C for one hour then 95°C for fifteen minutes. One chromosomal region was chosen to interval map based on the results of the chromosomal mapping. 2 μL of the forward primer corresponding to this region and 2 μL of the reverse primer (100 μM) were added to a mixture of 510 μL NEB 2X Taq Mastermix, 40 μL $MgCl_2$ and 486 μL nanopure water in a 1.5 mL tube10 μL of this mixture was added to every well of a 96 well plate. 0.5 μL of the mutant worm DNA was added to each well, the plate was centrifuged then placed in the thermocycler to undergo PCR under the same conditions as in chromosomal mapping. The DNA was digested using *DraI* in the same procedure as chromosomal mapping. Gel electrophoresis was used to analyze the DNA with the same preparation as chromosomal mapping. Recombination frequencies were calculated with the following equation:

$$\text{Recombination Frequency} = \frac{\text{Number of Wells Exhibiting Recombination}}{\text{Total Number of Chromosomes Present}} \times 100$$

Complementation Analysis

Known heterozygous hermaphrodites of M138 were mated with PD4792 males to produce heterozygous males. Said males were mated with known M136 heterozygous hermaphrodites. F1 generation offspring were screened for mutants. Complementation was said to have occurred if no mutants were observed.

Immunocytochemistry: MH4, MH27 and GFP Antibodies

A mutant plate with a large number of M136 or M138 embryos was washed with nanopure H_2O two to three times to remove live worms. The remaining embryos were removed by adding 1.0 mL of nanopure H_2O, rubbing gently and removing the H_2O to a 1.5 mL microfuge tube. The tube was centrifuged at 1000-1500 rpm for 30 seconds. The supernatant was removed, additional nanopure H_2O was added, tube was centrifuged again and the supernatant was again removed. 50 μL of the suspended embryos was removed to a glass slide and washed with 2% paraformaldehyde (pFA). 2% pFA was added, the slides were covered, the excess pFA removed and the slides placed in the humidity chamber for 20 minutes. The slides were frozen at -80°C. The coverslip was removed and the slide was transferred to 100% methanol on ice for three minutes. It air dried then was pre-incubated with TNB/10% NGS (0.10 M TrisHCl, 0.15 M NaCl, 0.5% BMP, 10% goat serum) in the humidity chamber for one hour. Selected primary antibodies were diluted in to 30 or 50 μL solutions of TNB/10% NGS. MH4 was used at a 1:3 dilution, MH27 was used slide and they were placed in the humidity chamber. After an hour, the slides were washed three times in TBS for five minutes each and

Table 1: Forward and Reverse Primers Used in SNP Mapping

Genetic Location	Primers	Genetic Location	Primers
I -19 FW	ATGCCAGTGATAAG GAACGG	I -19 RV	TCACATCCCTTGTCG ATGAA
I -12 FW	TCGAAATCAGGGAA AAATTGA	I -12 RV	ACGATTTTCGGGGA CTTTTT
I -6 FW	GTTTTCACTTTTGCC GGTGT	I -6 RV	TGAAGGCGCATATA CAGCAG
I -1 FW	AAAATATCAGGAAA GAGTTTCGG	I -1 RV	TTTAAAGATTAAGG GTGGAGCG
I 5 FW	ATCTGGCACCAAAT ATGAGTCG	I 5 RV	AATCTCGATTTTCAA GGAGTGG
I 13 FW	TCCTGGATAATCCCC AAAAA	I 13 RV	CCCTGCCATTGATCT TGTTT
I 14 FW	TTGAAATCCCCTTTA AAATCCC	I 14 RV	ACACTGGGTACCTG ACTCATGC
I 26 FW	ATTATTAACGGCCA CGGTGA	I 26 RV	CCCACACACTCTCA CCTTCA
II -18 FW	CCGAATTTTCAAAT GGATGC	II -18 RV	CCATTGGAATTGCA CACAAA
II -14 FW	CTGTGCTGTTGACG ATATTGG	II -14 RV	ATGTCTCATTGCAA AATTCGG
II -6 FW	TTGTGAGCTTATATC TCAGTTGTCG	II -6 RV	AGATTTGGTTAGAA ATATCACCGC
II 1 FW	TCAAAAACTTACAA TCAATCGTCG	II 1 RV	CCAGAAAATCTGCA CAGAAGG
II 4 FW	TTCTTCAAAAACTCT AGGTTCAGCA	II 4 RV	GGGGACGAAAACGG AGTTTG
II 11 FW	ACCGTTTAATAGGA TTATTTGGG	II 11 RV	AACTCTGCGGAATA ATTGATGG
II 16 FW	TTCCAGGTAATACA CATACAACTCC	II 16 RV	AAAAACACAAAGTT CAAAAACCC
II 22 FW	CCACTGGCTATAAG CTTTTCTAGG	II 22 RV	TAAGGATTTCAGGC TTTTAGGC
III -25 FW	TATCATCGAAATCC CGGAAA	III -25 RV	TTCGGACGGGAGTA GAATTG
III -19 FW	TCCCAATTTCCCTCT AAAAACC	III -19 RV	TTGAATTTGGACCAT TTTGAGG
III -12 FW	GAGGAACCAAATCT GGCGTA	III -12 RV	TGAAAACTTGGAAA ATCGGTG
III -7 FW	AATTTGAATCAGTG ACTTTTGGC	III -7 RV	TTTCTGCAAACATTT TTCTTCG
III -1 FW	AAAAATACATGTCT ACACAACCCG	III -1 RV	TTTCTTATCACTGTG CACTCTTACC
III 4 FW	AGCGTTAAAGTATC GGRRARRRCG	III 4 RV	TAAATTCATTTCAAA CAATCGAGC
III 12 FW	ATCAAGTTTCTGATT GCTCTTTCC	III 12 RV	AAAAACGTGATTTT TCAATTTTGC
III 21 FW	ACGAGGCTCACCAT CATCATCA	III 21 RV	GACATTACGGTAGA GGAGATGGA
IV -24 FW	TGATGGTGTGTCTGC GTACC	IV -24 RV	AGAGCTGGAGAGCA CGGATA

IV -24	TGATGGTGTGTCTGCGTACC	IV -24 RV	AGAGCTGGAGAGCACGGATA
IV -16 FW	CGCATAAATCCAACGTTCTCTG	IV -16 RV	AATCCATAAGTTTCGTGTTGGG
IV -7 FW	ACTCGGCATCCTCACGC	IV -7 RV	GTTGAAAATTTTTTCATAGCTATCATC
IV 1 FW	AAAATGGGAAGCGTACCAAA	IV 1 RV	TGCTTGTAGCGTTTCCAAGA
IV -5 FW	TGCTGAAATATTGGAAAATTGAGG	IV -5 RV	TTATATCGTCGAGGAGGTTAGAGG
IV 8 FW	GACACGACTTTAGAAACAACAGC	IV 8 RV	TGGTATGGAGTCCCTATTTTGG
IV 12 FW	TCGAATTGTTGTGTTTCTTTTGA	IV 12 RV	AAATTTCCAATTGTTCAAAGCC
IV 14 FW	TCGAATTGTTGTGTTTCTTTTGA	IV 14 RV	TTCCAATTTTCTCGGTTTGG
V -17 FW	TTTCGGAAAATTGCGACTGT	V -17 RV	CGCGTTTTGGAGAATTGTTT
V -13 FW	TCATCTGTTATTTCGTCTCTTGC	V -13 RV	CGGTAATAATATGCTTTGTGGG
V -5 FW	GAGATTCTAGAGAAATGGACACCC	V -5 RV	AAAAATCGACTACACCACTTTTAGC
V 1 FW	AGAAATGATCCGATGAAAAAGC	V 1 RV	CCGATAGTGTTCATAGCATCCC
V 6 FW	CAAATTAAATATTTCTCAAAGTTTCGG	V 6 RV	ACATAAGCGCCATAACAAGTCG
V 10 FW	TAAAGCCGCTACGGAAATACTC	V 10 RV	ATTTTTCTCCCTAATTCCAGGTG
V 13 FW	CATTCATTTCACCTGTTGGTTG	V 13 RV	TCGGGAAGATAATCAAAATTCG
V 18 FW	GAAATTCAAATTTTTGAGAAACCC	V 18 RV	TTCAGACCATTTTTAGAATATTCAGG
X -17 FW	ATATGTGAGTTTACCATCACTGGG	X -17 RV	ACGTTTTGAAAAATTTGGTTGC
X -8 FW	CCAAAACGGCCAAGTATCAG	X -8 RV	TTGCACTCTTTCTCCTTCCG
X -4 FW	AAGTGTTCAATGATTTTGTCTAATTG	X -4 RV	TGACAGGAGAATACTTTTGAAGG
X 2 FW	AGCAACAAACAATGCAACTATGG	X 2 RV	TAAACAAGAGGGTACAAGGTATCG
X 8 FW	TTAAAACCATACAATTCTTCTCAGC	X 8 RV	GAATTCCCAATCAACAGAGAGC
X 11 FW	ACTGTTTACCGCGTCTTCTGC	X 11 RV	CCGTGTATATAAGAAAATGTGTTCG
X 17 FW	GCTGGGATTTTGAAGAGTTGTT	X 17 RV	CAGTGAATCATCCGTTGAATTT
X 23 FW	CAAATACCAAGTTGATCGTGG	X 23 RV	TTGTTGCAATTAAATCAAACGG

Table 2: Expected Hawaiian and Bristol Digests Using *Dra*I Restriction Enzyme

SNP Region	N2 Digest	Hawaiian CB4856 Digest
I -19	354, 146	500
I -12	503, 72	377, 126, 72
I -6	395, 144	538
I -1	325, 134, 41	459, 41
I 5	494	365, 129
I 13	445	295, 151
I 14	236, 99, 78	335, 78
I 26	360, 114, 27	474, 27
II -18	263, 112	375
II -14	345	236, 109
II -6	516	387, 129
II 1	373, 121	494
II 4	224, 117, 124, 44	340, 124, 44
II 11	484	352, 132
II 16	500	368, 132
II 22	365, 119	484
III -25	206, 189	395
III -19	342, 78, 76	272, 78, 76, 70
III -12	368, 105	473
III -7	239, 85, 27	196, 85, 43, 27
III -1	486	354, 132
III 4	355, 142, 30	497, 30
III 12	339, 156	495
III 21	273, 137, 78	200, 137, 78, 73
IV -24	301, 128, 71	429, 71
IV -16	187, 304	491
IV -7	498	250, 248
IV 1	295, 124	419
IV -5	376	300, 76
IV 8	313, 77	390
IV 12	284, 162, 52	327, 119, 52
IV 14	241, 108, 78, 48	319, 108, 48
V -17	307, 87, 79	386, 87
V -13	288, 167	455
V -5	454	307, 147
V 1	435, 70	300, 135, 70
V 6	500	348, 152
V 10	475	288, 187
V 13	282, 205	487
V 18	324, 164	488
X -17	540	321, 219
X -8	422, 72, 40	326, 96, 72, 40
X -4	169, 54, 51, 35, 22	223, 51, 35, 22
X 2	409, 133	542
X 8	341, 126	467
X 11	318, 191, 37	509, 37
X 17	409, 34	302, 107, 34
X 23	358, 134	492

at a 1:100 dilution and GFP was used at a 1:200 dilution. Excess TNB/10% NGS was removed from slides and selected primary antibodies were added and placed in the humidity chamber overnight. The slides were washed three times in TBS buffer (150mM NaCl, 10 mM Tris pH 8.0) for five minutes each then excess was removed. Secondary antibodies, one for GFP one for either MH4 or MH27, were diluted to 1:200 with TNB/10% NGS, 200 μL was added to each the excess was then removed. 10 μL of mounting media was added, the slide was covered and sealed. The slides were viewed under a Nikon Eclipse TE2000-U microscope and photographed.

Genetic Cross of SM1493 and M136

PD4792 males were mated with known heterozygous M136 hermaphrodites (Figure 7). F1 males were mated with SM1493 rolling hermaphrodites that possessed *myo-2*::GFP::H2B. After 24 hours the rollers were moved to individual plates. The offspring of this mating that exhibited *myo-2*::GFP and the rolling phenotype were moved to individual plates. Their progeny was screened for mutant organisms possessing *myo-2*::GFP. If mutants were present, rolling worms with *myo-2*::GFP::H2B were moved to individual plates. These were screened for possible mutants that did not possess *myo-2*::GFP, but appeared to be very small, unmoving, in odd orientations and possibly with *myo-2*::GFP::H2B. Worms with this appearance were placed on a slide, viewed under the Zeiss Axiovert 100 microscope and photographed extensively if possessing muscle cell nuclei.

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